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ICP-MS-based competitive immunoassay for the determination of total thyroxin in human serum

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To further expand the range of analytes that can be detected by using ICP-MS coupled with bioanalytical methods, we have employed a new separation system based on the highly active surface streptavidin and biotinylated monoclonal antibody (McAb) in a competitive immunoassay followed by ICP-MS detection. Specifically, we have demonstrated its application for the determination of total thyroxine (T_4) in human serum using Eu^{3+} as a label. In this method, streptavidin immobilized to the pre-coated bovine serum albumin (BSA)-biotin on the microwells showed a significantly higher binding capacity for the biotinylated anti-T_4 monoclonal antibody. Total T_4 was quantified by measuring the Eu^{3+} intensity with ICP-MS after using 1% HNO_3 to extract Eu^{3+} from the bound fraction of the immunocomplex. The detection limit was 7.4 ng mL^{-1} with a sample volume of 25 μL. Total T_4 values obtained by this procedure agreed well with those obtained by a chemiluminescent immunoassay method. Both inter- and intra-assay precisions were below 10%. The results indicate that a general competitive ICP-MS-based immunoassay scheme may be possible.

Introduction

The competitive immunoassay has been well established for measuring concentrations of antigens and, more commonly, small molecules such as drugs and some hormones.[1–4] Most competitive immunoassays are solid-phase immunoassays where microwells or tubes, *etc.*, are used as carriers of an immunoreagent. The typical immobilization of an antibody/antigen in competitive immunoassays includes both direct and indirect means. For the former, a primary antibody or antigen is absorbed on the surface of the solid phase, and a high uniformity of immobilized surface reagents is required to ensure a reproducible and accurate measurement. The indirect immobilization method, having the advantages of improving precision and reducing the amount of antigen or primary antigen-specific antibody, has been extensively employed in immunoassays.[5–8] However, in our development of total serum thyroxin determination using an immobilized second-antibody as the separation agent, we observed a significant measurement bias caused by a matrix interference when the second-antibody immobilized had a relatively low binding capacity for the primary anti-T_4 McAb. To overcome these limitations, we employed a new separation system based on the highly active surface streptavidin and biotinylated anti-T_4 McAb for the determination of total T_4 in a competitive format.[9]

The effectiveness of immunoassay as a diagnostic tool derives from not only the structural specificity of the antigen-antibody reaction, but also the detectability of labeled reagents. During the past decades, immunoassay methods have been developed firstly using radioisotopic labels and then non-isotopic labels, such as enzymes and luminescent materials, in order to avoid the well-known limitations associated with radioisotope labels. Consequently, the choice of label technique leads to different means of detection, such as radioisotopic, colorimetric, fluorescent and chemiluminescent detection.[10] These methods still play important roles in many important fields.[11–13]

In order to expand detection methods for immunoassays, electrothermal atomic absorption spectrometry and ICP-MS have also been reported as the endpoint detection.[14–18] ICP-MS is an outstanding method for the determination of inorganic elements because of its speed, multi-component analysis ability and amenability to automation. Therefore, it might be considered as a good detector for use in immunoassays if a suitable metal is chosen as a label. In this study, the concentration of total T_4 in the sample was quantified by the ability of T_4 in serum to inhibit the binding of the Eu^{3+}-labeled T_4–BSA conjugate to the biotinylated anti-T_4 McAb. Eu^{3+} was finally determined using ICP-MS. The results of the present method indicate that a general competitive ICP-MS based immunoassay scheme may be possible.

Experimental

Instrument

An Elan 6000 ICP-MS (PerkinElmer/SCIEX) equipped with a cross-flow nebulizer was used. The parameters of the instrument for Eu measurement were optimized daily. The parameters were as follows: nebulizer gas, 0.94 L min^{-1}; lens voltage, 8.25 V; and rf power, 1200 W. Peak hopping mode was used in the experiment. The chromatographic separation system, including a Model EP-1 Econo Pump and a Model EM-1 Econo UV monitor, is produced by Bio-Rad. T_4 concentrations for the comparison study were obtained from an ACS:180 automated chemiluminescent immunoassay system.

Reagents

Anti-T_4 McAb (Clone No. 6901) was obtained from Medix Biochemica, Finland. Streptavidin and biotinamidocaproate *N*-hydroxysuccinimide ester (BAC-NHS) were products of Sigma. Purified water (18 MΩ cm) was used throughout the experiment (Beijing ShuangFeng purity water equipment factory). *N*-[*p*-isothiocyanato-benzyl]-diethylene-triamine -N^1,N^2,N^3,N^3-tetraacetate-Eu^{3+} (DTTA-Eu^{3+}) was from Tianjing Radio-Medical Institute (Tianjing, China). Microtitre strips were obtained from NUNC Co., Denmark. Super-purity

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HNO_3 was obtained from Beijing Chemical Reagents Institute (Beijing, China).

The following buffers were used. Coating buffer: 100 mmol L^{-1} sodium carbonate buffer (pH 9.0), containing 0.9% NaCl and 0.04% NaN_3. Blocking buffer: 50 mmol L^{-1} Tris-HCl (pH 8.0), containing 0.9% NaCl, 0.04% NaN_3 and 1% BSA. Assay buffer: 100 mmol L^{-1} Tris-HCl (pH 8.4), containing 0.25 mg mL^{-1} 8-anilino-1-naphthalene sulfonic acid (ANS), 4.0 mg mL^{-1} sodium salicylate, 0.1% BSA, 0.04% NaN_3, 0.9% NaCl and 0.04% Tween 20. Wash buffer: doubly-distilled water containing 0.04% Tween 20, adjusted to pH 8.0 with Tris solution.

Preparation of T_4–BSA conjugate and europium labeled T_4–BSA

The details for the preparation of T_4–BSA conjugate and tracer were described in our previous report.[19] Briefly, 0.6 g of L-T_4 was dissolved in 25 mL methanol and reacted with HCl gas to obtain 0.39 g thyroxine methyl. Then, 0.3 g of thyroxine methyl in 40 mL *N*,*N*-dimethylformamide (DMF) was added to the BSA solution to form the T_4-BSA conjugate using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling reagent. The molecular ratio of T_4 to protein was 4:1, which was determined by measuring the absorbance of the conjugate solution (0.01% in distilled water) at 280 and 342 nm.

2 mg of T_4–BSA in 50 mmol L^{-1} sodium carbonate buffer (pH 9.5) was reacted with 1 mg DTTA-Eu^{3+}. The unreacted DTTA-Eu^{3+} was separated from the tracer by size-exclusion chromatography on a 1.5 × 45 cm column of Sepharose-6B (Pharmacia, Uppsala, Sweden). The eluting agent was 50 mmol L^{-1} Tris-HCl buffer, pH 7.7, containing 0.9% NaCl and 0.05% NaN_3.

Biotinylation of anti-T_4 McAbs and BSA

40 μL of BAC-biotin solution, freshly prepared before use by dissolving 4.5 mg of succinimidyl-(biotinamido)hexanoate-biotin in 2 mL of DMF, were added to 2 mg of McAbs solution. The contents were then mixed and incubated for 30 min at ambient temperature. After that, the mixture was dialyzed for 48 hours against 1000 ml of 0.1 mol L^{-1} Tris-HCl buffer (pH 8.0) containing 0.9% NaCl and 0.05% NaN_3.

BSA was biotinylated with a similar procedure as described above except the concentration of BAC-biotin was increased to 12 mg mL^{-1}.

Preparation of surface streptavidin

Microwells were first activated by incubation with 200 μL of coating buffer containing 1 μg of biotinylated BSA, and the coating procedure was allowed to proceed for 24 hours at ambient temperature. The microwells were washed twice, and 200 μL of 50 mmol L^{-1} Tris-HCl buffer, containing 0.8 μg mL^{-1} of streptavidin, 1% BSA, 0.9% NaCl and 0.04% NaN_3, were added followed by incubation for 24 h.

Immunoassay protocol

Firstly, the microwells were washed twice, and then 25 μL of T_4 standards or serum samples were pipetted into the microwells. 35 ng of T_4-BSA-Eu^{3+} in 50 μL assay buffer were added, followed by addition of 50 ng biotinylated anti-T_4 antibodies in 100 μL assay buffer in each microwell. After that, the mixture was incubated for 6 hours at ambient temperature. The microwells were then washed 6 times with washing buffer. Finally, 200 μL aliquots of 1% HNO_3 were pipetted into each well. After 3 min, the samples to be analyzed were introduced into the ICP-MS instrument by means of a peristaltic pump at a flow rate of 1mL min^{-1}.

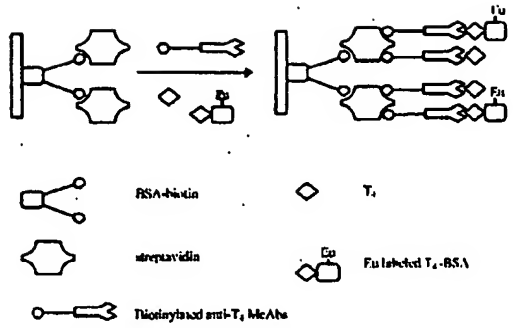

Fig. 1 Schematic diagram of total T_4 determination using the ICP-MS based immunoassay.

Results and discussion

Optimization of immunoreaction conditions

In the development of total T_4 determination, we firstly employed the surface second antibody as the separation agent. A measurement bias caused by matrix interference, however, was observed due to the relatively low binding capacity of the second antibody for the primary anti-T_4 McAb.[9] Since the unique characteristics of biotin-(strept)avidin interaction make it ideal as a general bridge system in many diverse applications,[20] the use of the highly active surface streptavidin and biotinylated anti-T_4 McAb for total T_4 measurement in competitive format was proposed to eliminate the matrix interference. The schematic diagram based on the streptavidin surface immobilization is shown in Fig. 1. BSA-biotin conjugate was firstly coated on the microwells by passive adsorption. Streptavidin was then immobilized onto the microwells *via* specific binding to the surface biotin moiety. Because of the tetrameric binding feature of streptavidin and the controllable biotinylation of BSA, the surface streptavidin prepared in this method showed a significantly higher binding capacity for the biotinylated anti-T_4 antibody.[9]

In principle, T_4 can be labeled with DTTA-Eu^{3+} since T_4 contains an -NH_2 group for coupling. However, this requires careful optimization for separating the labeled T_4 from the free DTTA-Eu^{3+}. Using T_4-BSA instead of T_4 obviates such a problem, because T_4-BSA-DTTA-Eu^{3+} can be separated easily from the free DTTA-Eu^{3+} by routine size-exclusion chromatography. In addition, the T_4-BSA conjugate can be labeled extensively with DTTA-Eu^{3+} due to the abundant -NH_2 groups available in BSA, leading to an enhanced specific response in the T_4 immunoassay.

The immunoreactions between biotinylated anti-T_4 McAb and T_4 in the standard/sample or the europium-labeled T_4-BSA conjugate mainly occurred in liquid solution, and then the immune complex was specifically trapped by the surface streptavidin and isolated from the free tracer by the washing step. In addition, coating with pre-coated BSA-biotin, instead of directly coating the straptavidin onto the surface of microwells, greatly reduced the consumption of streptavidin.[9] As a result, streptavidin immobilized to the pre-coated BSA-biotin can become a good bridge system in immunoassay at very low cost.

In the competitive assay, Eu^{3+}-labeled T_4-BSA competes with T_4 in samples for binding to a limited amount of biotinylated anti-T_4 McAbs. In order to obtain the optimal incubation time in this system, biotinylated anti-T_4 McAbs, T_4 in the standard, and the Eu^{3+}-labeled T_4-BSA solution for immunoreaction were incubated at room temperature for 6, 12 and 24 hours. Fig. 2 shows a plot of the resulting binding ratio B/B_0

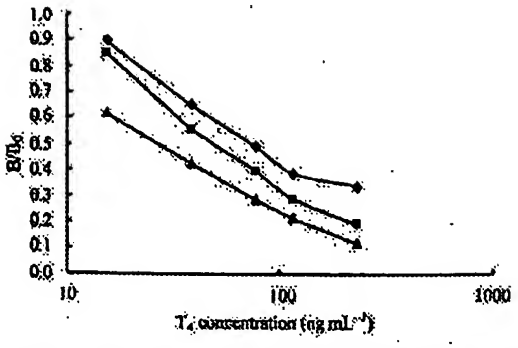

Fig. 2 The effect of incubation time for the immunoassay: ▲, incubation 6 hours at room temperature; ■, incubation 12 hours at room temperature; and ◆, incubation 24 hours at room temperature.

(B_0 = signal obtained when zero analyte is present in the sample, B = analyte signal) as a function of incubation time.

At first, the blank sample gave the highest signal since there is no competition between the Eu^{3+}-labeled T_4-BSA and T_4 in the samples for binding to a limited amount of biotinylated anti-T_4 McAbs. As the reaction of biotinylated anti-T_4 McAb with T_4 in the sample and Eu^{3+}-labeled T_4-BSA proceeded, both free and Eu^{3+}-labeled haptens followed the behavior expected of competitive assays: the increase in the amount of T_4 in the sample led to a decrease in signal. As also can be seen from Fig. 2, as the incubation time increased from 6 to 24 hours, this led to an increase of the B/B_0 value for each calibration point. However, prolonging the incubation time up to 12 and 24 hours gave a very high B/Bo value in the low T_4 concentration range, which led to decreased analytical sensitivity. A 6 hour incubation time was preferred in the assay. It should be noted that the incubation time may be further shortened to 2–3 hours by gentle shaking at room temperature.

Optimization of ICP conditions

Some problems occur in ICP-MS due to polyatomic interference. Removal of the interfering species before introduction into the plasma can eliminate the interference. In our experiments, Eu^{3+} was employed as the label. Eu has two isotopes with abundances of 47.8% (^{151}Eu) and 52.2% (^{153}Eu), respectively. The spectral interference in Eu determination results from Ba ions in the solution, since $^{137}BaO^+$ (153) and $^{135}BaO^+$ (151) have the same mass number as ^{153}Eu and ^{151}Eu. However, interference of Ba ions can be easily controlled to the minimum by using high purity grade regents and 18 MΩ cm purified water. Although all Eu isotopes are suitable for our application, ^{153}Eu was chosen because of its relative abundance (52.2%) compared to ^{151}Eu (47.8%).

HNO_3 is the first choice of inorganic acid to be used in ICP-MS because it is less likely to form polyatomic substances that may cause interference in the determination of a specific element. In this ICP-based immunoassay, HNO_3 was employed to extract the Eu^{3+} from the immune complex, followed by delivery of the extracted solution to the ICP-MS. Fig. 3 shows that the maximum signal of Eu was observed using 1% HNO_3 as eluent. Probably the highest atomization was achieved by using 1% HNO_3, and it is possible that a HNO_3 concentration of 2.5% or higher may affect the atomization process of the ICP-MS, resulting in suppression of the analytical signal.

Internal standardization is one of the useful techniques for correction of instrument parameter fluctuation in ICP-MS. ^{187}Re served as the internal standard in this method. The final concentration of ^{187}Re was 1 ng mL^{-1} in 1% HNO_3.

For the determination of total T_4 by ICP-MS, dissociation of Eu^{3+} from the solid phase antibody after immunoreaction is a key step. In a similar way to the dissociation process of the time-resolved fluorescence method, in which Eu^{3+} can be dissociated from the solid-phase antibody at pH 3–3.5 in a buffer containing 0.1–0.2% Triton X-100, β-diketone and trioctylphosphine oxide (TOPO),[21] we carried out a simple dissociation step by using 1% HNO_3.

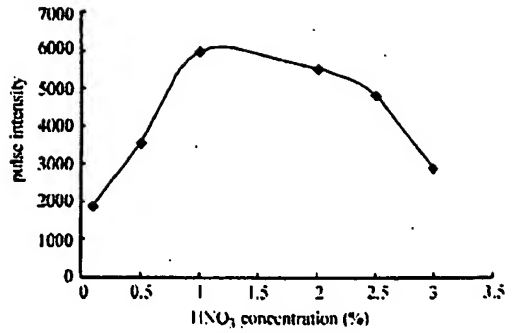

Fig. 3 Effect of HNO_3 concentration after immunoreaction: T_4 concentration, 77.7 ng mL^{-1}; and flow rate of pump, 1 mL min^{-1}.

Analytical characteristics

T_4 has been proven to be of high value in the diagnosis of thyroid diseases. In the bloodstream, T_4 is predominantly bound to the carrier proteins, thyroxine-binding protein and albumin. The normal range for total T_4 in serum is generally 50–120 ng mL^{-1}. Up to now, several methods have been reported for the detection of total T_4 in human serum.[22–26] A typical standard curve and precision profile (n = 4) for the present assay is shown in Fig. 4. The detection limit of the assay was 7.4 ng mL^{-1}, defined as the T_4 concentration corresponding to the mean Eu signal intensity of a zero standard (n = 12) minus three standard deviations. The working range was up to 233 ng mL^{-1}. Obviously, the ICP-MS-based method is a suitable alternative that can meet the clinical requirements for total T_4 detection.

Both intra- and inter-assay precisions of the measurement (n = 4) of three T_4 standards at 15.5, 77.7 and 116.6 ng mL^{-1} were studied. The inter-assay RSD were 8.2%, 5.5% and 7.4%, and the intra-assay RSD were 8.0%, 5.3% and 6.1%, respectively.

The dilution linearity was assessed, by assaying T_4 samples after serial dilution with T_4-free human serum. The results, shown in Table 1, suggest a good agreement between the expected and the observed values.

20 serum samples were assayed. The results obtained from the present method were compared with those obtained from a chemiluminescent immunoassay method. A relatively good correlation, as shown in Fig. 5 (r^2 = 0.9529), was obtained between the two methods.

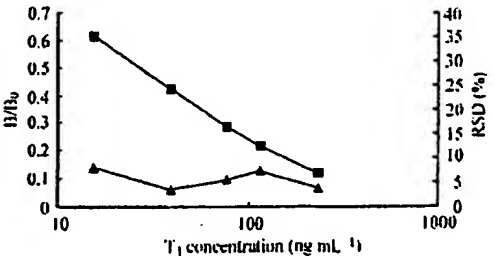

Fig. 4 A typical standard curve and of one total T_4 determination: ■, standard curve; and ▲, precision profile.

Table 1 Sample dilution with T_4-free human serum

	Dilution factor				
	Original concentration/ ng mL^{-1}	2	4	8	16
Expected	215	107.5	53.8	26.9	13.4
Measured		105.2	51.6	28.4	11.5
Expected	146	73	37.5	18.5	9.3
Measured		74.1	36.4	17.1	11.6
Expected	92	46	23	11.5	
Measured		44.2	20.8	13.5	

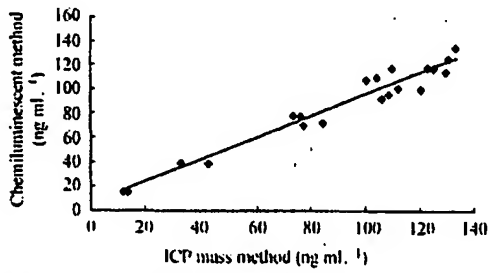

Fig. 5 Correlation between proposed method and a chemiluminescent method.

Conclusion

We have demonstrated that a highly active surface streptavidin-biotin based separation system in combination with ICP-MS can be adapted to function in a competitive immunoassay. This separation system is employed to avoid the bias of the measurements due to the matrix difference. Besides the application of surface streptavidin in a competitive immunoassay, as described in this work, the surface streptavidin can also be applied to non-competitive immunoassays at very low cost. The advantages of utilizing ICP-MS as the detection method for metal-labeled large and small molecules lie in its high sensitivity and multi-analyte detection capability. The successful combination of immunoreaction with ICP-MS shows promise for the simultaneous detection of a wide variety of important clinical analytes in one run, by using different metal tags.

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